Antimicrobial and Cytotoxic Activities of Extracts from *Laurus nobilis* Leaves

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

King Abdullah University of Science and Technology

Thuwal, Kingdom of Saudi Arabia

May 29, 2011

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ABSTRACT

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The cytotoxic activity and antimicrobial properties of crude extracts from *Laurus nobilis* were investigated. With the use of the organic solvents, methanol and ethanol, crude extracts were obtained. To determine the availability of active bio-compounds, an analysis using liquid chromatography was conducted. The crude extract was also tested for antimicrobial activity. The disc diffusion method was used against the bacterium *Escherichia coli*. The results showed a weak antimicrobial activity against *E. coli*. For cytotoxicity testing, the crude extract was studied on four cell-lines: human breast adenocarcinoma, human embryonic kidney, HeLa (human cervical adenocarcinoma), and human lung fibroblast. From the alamarBlue® assay results, the extracts most potently affected the cell-lines of human breast adenocarcinoma and human embryonic kidney. Using the lactate dehydrogenase (LDH) assay, an effect on human embryonic kidney was most prominent. With these findings, a suggestion that the crude extract of *Laurus nobilis* may have antiproliferative properties is put forth, with the possibility of this mechanism being induction of apoptosis with the involvement of Nuclear Factor Kappa κB (NF κB).

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LIST OF ABBREVIATIONS

BA	breast adenocarcinoma
DMSO	dimethyle sulfoxide
EK	embryonic kidney
ESI	electrospray ionization
Hl	HeLa
HL	HeLa
LB	Luria Bertani
LC	liquid chromatography
LF	lung fibroblast
LDH	lactate dehydrogenase
MS	mass spectrometry
UV	ultraviolet

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I. INTRODUCTION

Plant-Derived Natural Products

One of the large fields in human history is the development of natural products from traditional plants. Indigenous plants have always been utilized in different areas of our lives. Many of them are used as spices, perfumes, flavorings, and even poisons. They are also used in remedies like tinctures, teas, poultices, powders, and other herbal formulations[1]. They have been integrated into many people's upbringing due to their influence and importance. Many of these ancient traditional practices involved Indians, Chinese, Egyptians, Babylonians, Greeks, Romans, Persians, during medieval history and also the modern era. What is completely fascinating is that a lot of these ancient practices are still being used in many places nowadays. For example, there are many recipes that are used for headache treatment, like rubbing the head with apple vinegar or drinking the tea of sweet violet.

The continuing interest in natural resources as a potential area for identification of novel bioactive molecules is impressive. The area has developed from practical knowledge that was passed on through generations and was based on acquired experience, to having people dedicated to describing and characterizing the plants superficially, to use of more chemical and biological methods based on the huge advancement in extraction and isolation methods that currently are used in obtaining new and beneficial compounds[2].

The process of isolation and purification of bioactive natural products started back in the early 19th century, when morphine was isolated from the dried latex of opium (*Papaver sominiferum*) by F. W. Serturner[3]. But before that came there were many accounts of using plants as therapeutic agents. Among the best example is the Buddhist, which included the use of many different medicinal plants, and provided one of the reasons to cultivate plants and regard them in a scientific manner[3]. Also, among the many early discoveries of drugs derived from natural resources include cardiotonics in foxglove, penicillin in mold and salicylic acid in willow bark[4].

Like all living things, plants produce a lot of organic compounds that have different roles in their metabolic pathways. These compounds are classified according to their function into “primary metabolites” and “secondary metabolites”. However, it is not always easy to distinguish between these two classes based on their precursor molecules, chemical structures, or biosynthetic origin. So with the lack of clear distinctions based on structure or biochemistry, the differences in their functional properties is used as a distinguishing feature. Primary metabolites are essential to the plants’ metabolic processes and nutrition, and they could be found in the entire plant kingdom. Secondary metabolites, which are also natural products, are commonly found throughout the entire plant kingdom in low concentrations. Most of them show no effect on the development and the growth of plants but they can affect the ecological relationship between the plant and its surroundings[5].

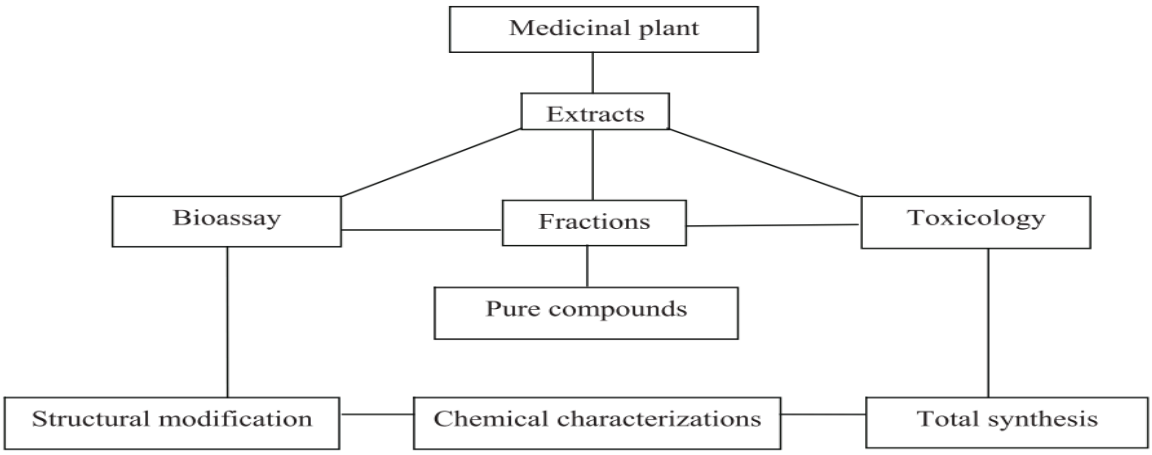

Figure 1: Method of obtaining bioactive molecules from plants[3].

One of the major downsides to natural product research (Figure 1) has been difficulties in determination of their complex structures and biosynthetic pathways. Plant biologists have generally considered them biologically insignificant because they were produced in small amounts, so they received little attention. However, studying novel phytochemicals has attracted the attention of organic chemist since the 1850s, which has led to the shaping of organic chemistry, as we know it today[5].

This interest in natural products has included many areas like polymers, oils, waxes, and drugs. With knowledge of their biological properties, it allowed expansion of the search for novel medicines such as antibiotics, insecticides, and herbicides. With information on the highly varied biological effects induced by natural products, it gave way to reassessing the current standing of these compounds in plants, specifically regarding with their interaction with their ecology[5].

Despite sporadic claims over the years that interest in natural products is waning, still, natural products have a lot of advantages. These include their composition, weight, size, functional groups, and architectural and stereochemical complexity, that make the compounds more likely biologically useful. Such compounds could function as inhibitors or activators for certain proteins that they could bind to in specific biological systems. To show their value, 61% of 877 small organic compounds that were introduced as drugs from 1981-2002 are of natural product sources, their derivatives or their mimetics[6].

Also, an important consideration in the field of natural products is that they are considered "privileged structures" derived evolutionary for their ability to bind to biological macromolecules, making them useful templates for the synthesis of novel, biologically active, natural product-like derivatives. With the availability of the right methods to study the chemical structure and biological activity of the optimized products, natural product-based drug discovery and development has become multidisciplinary[7]. The typical process of isolating compounds from medicinal plants includes the work of botanists, ethno-botanists, ethnopharmacologists, or plant ecologists[8]. Also, the input that is given to scientists by people who have practical knowledge in the use of these plants should be respected and considered.

One of the difficulties in the area of natural product research has been the limited availability of source material. Many plants will be found in small quantities and their biomass is limited. For this reason, new genomic methods and the development of engineered biosynthetic pathways were developed to help advance the field forward

and enable large-scale production of specific natural products. Since directly isolated natural products have only a small chance to be the actual future treatment for any given disease, utilizing them as lead compounds for developing analogs using combinatorial biosynthesis and/or combinatorial chemistry, has resulted in compounds with optimized pharmacological properties. Such approaches can be used to overcome the difficulties in this area[3].

During the 20th century there has been move to replace plant-based drugs with drugs that are derived from microbes and chemically synthesized novel compounds[9]. However, the introduction of small-molecule natural products that are derived from plants for therapeutic use, like acarbose, capsaicin, docetaxel, galantamine, and paclitaxel has continued[10,11,12]. From progress to date, it seems that research into plant-derived drugs is going to continue to grow.

As mentioned above, many plants are currently being studied for the presence of novel bioactive molecules. One of the advantages of plants compared to other organisms is the ethno-botanical knowledge that has been passed down and acquired through generations of herbal medicinal use.

The use of traditional medicine is common in Saudi Arabia. It is applied to treat many types of ailments that affect the human health. Despite its popularity, little is known scientifically about the actual mechanisms, which result in the effectiveness of these treatments. It has been estimated worldwide that 75% of the 120 biologically active

plant-derived compounds were discovered following the study of traditional medicinal plants. Between 10 and 60% of plant-derived drugs are anticancer compounds[13].

As has been mentioned repeatedly, the methods utilized for drug discovery are numerous. They require methods in bioassays, chemical isolation, synthetic chemistry, combinatorial chemistry, and molecular modeling and activity testing. Despite this technology, which has been funded, developed and used by pharmaceutical companies and organizations, the interest in isolation of plant-derived bioactive molecules, specifically with medicinal plants, is still expanding. As of 2001 and 2002, it was estimated that a quarter of best-selling drugs worldwide came from natural products or were derived from natural products[8].

To emphasize this point, there have been four new drugs introduced to the U.S. market recently that have been derived from medicinal plants[1]. Arteether (trade name Artemotil®), which is an anti-malarial drug, was derived from a traditional Chinese plant called *Artemisia annua* that contains artemisinin, a sesquiterpene lactone from which the drug was derived[2]. Galantamine (trade name Reminyl®) was found in Russia in the early 1950s in a plant called *Galanthus woronowii* and is now approved for use in treatment of Alzheimer's disease[3]. Nitisinone (trade name Orfadin®) is used against a rare inherited disease called tyrosinaemia. Nitisinone is modified from an herbicide derived from natural products called leptospermone. The plant that nitisinone is derived from is called *Callistemon citrinus*[4]. Tiotropium (trade name Spiriva®) which is used in the treatment of chronic obstructive pulmonary disease (COPD). Tiotropium is an

anticholinergic bronchodilator, which is inhaled. Anticholinergic bronchodilators are based on ipratropium, which is derived from atropine. The plant that tiotropium is isolated from is called *Atropa belladonna*[8].

The long process required for drug-development should be noted. For example, paclitaxel (Taxol®) was extracted from *Taxux brevifolia* in 1971 and identified and reported to have cytotoxic activities[14]. But Taxol® was only approved at the end of 1992 as a cancer chemotherapeutic agent. Usually the development and commercialization of new drugs takes at least a decade and costs between $400 and $800 million US dollars. This process has become even more difficult over the last few years. As shown in (Figure 2) and (Table 1), the long and difficult processes involved in developing new drugs are illustrated[13].

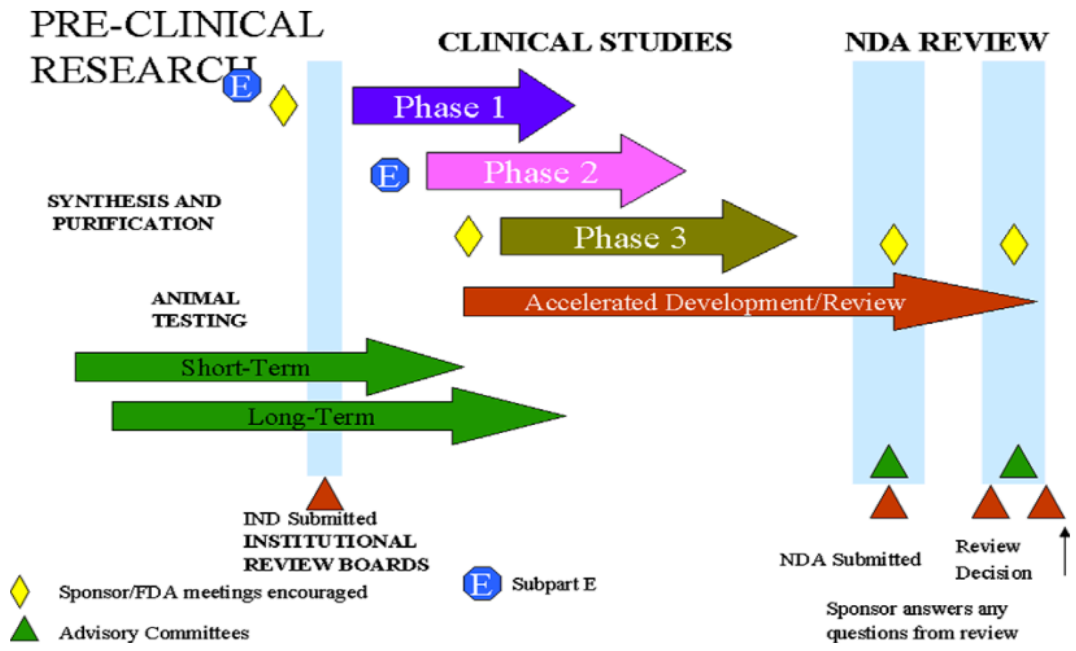

Figure 2: Outline for development of new drugs[13].

	Usual Range of Time Required (years)	Approximate Mean Time Required (years)
Stage of Development		
1. Project Formation to IND Filing	1.5 to 3.5	2.5
2. Phase I Clinical Studies	0.5 to 1.5	1.0
3. Phase II Clinical Studies	1.0 to 5.0	3.0
4. Phase III Clinical Studies and Preparation of NDA	1.0 to 5.0	3.0
5. FDA Review of NDA	1.0 to 5.0	2.5
Totals	5.0 to 20.0	12.0

Table 1: The amount of time required developing new drugs in the U.S.[13] IND: Investigation New Drug. FDA: Food and Drug Administration.

Anticancer Drug Discovery

It is estimated that around 6 million cases per year of new reports of cancer patients are reported. In general, cancer refers to the unnatural progression of normal proliferation processes of cells. The search for cancer treatments using natural resources dates to the Ebers papyrus in 1550 BC, at least. However, the actual period of scientific interest in cancer treatment begins with Hartwell and colleagues in the late 1960s with the application of podophyllotoxin[15].

Because of the importance of the disease, the search for novel anticancer treatments is a major area of research in drug discovery. Despite that, novel compounds under

consideration for anticancer therapy development face many challenges. In particular, it is easier in other therapeutic areas such as inflammation to predict the likely clinical success rate of new drugs based on a variety of animal model systems. This assumes that the pharmaceutical feature of the molecules studied can be designed or modified to yield better analogues that will affect the toxicity, activity or solubility of the drug. However, models like the ones commonly used in other disease processes, such as infectious diseases, are lacking in cancer treatment. For this reason, the attitude that is used in oncological drug development is: (i) 'fail fast', which means quickly identify and discard the compounds that have a small change in succeeding, and (ii) 'advance smartly', which means to carefully move forward with those compounds likely to provide rewarding results[16]. Also, a huge issue in this area is the toxicity of most cancer treatments. Most cancer treatments are not specific to cancer cells and also kill normal cells. Another problem is the drug resistance that often happens after some time, despite the use of combinatorial treatments involving multiple drugs[15]. Another problem that should be noted is that the number of cancer treatments that fail to produce effective results actually outnumber the ones that have been shown statistically to be effective. This suggests that the methods used for developing novel compounds as anticancer drugs for commercial production needs adjustment. New and better approaches for anticancer drug development is needed, with the aid of more relevant *in vivo* tumor models[17].

Plant derived compounds that are currently in clinical use as anticancer agents are classified into four classes. First are the vinca (or *Catharanthus*) alkaloids used to block mitosis metaphase arrest. Vinca alkaloids work by binding to tubulin, which leads to its depolymerization. Second are the epipodophyllotoxins, which function by binding to tubulin and cause the DNA strand to break during the G_2 phase of the cell cycle through inhibition of DNA topoisomerase II. Third are the taxanes, which act by binding to tubulin without affecting its assembly or depolymerization. Finally, are campotothecins, that function by selectively inhibiting to topoisomerase I which is involved in the process of cleaving and reassembling DNA. Since 2002, the taxanes and campotothecins make up one-third of the global anticancer drug market[8].

In summary, focusing more on natural resources for developing safe, economic, and site-specific anticancer drugs will lessen the challenges, and hopefully enable us to better understand this ailment that has plagued the lives of so many people.

Antimicrobial Drug Discovery

Looking for new compounds with antimicrobial activity is another major challenge. Using plant resources is a major potential approach in this area because of the increasing numbers of bacteria resistant to currently available antibiotics. The search for new sources for antimicrobial drugs is one of the major challenges to human health in this century[18].

Various reports between 2000 and 2008 have shown that there are more than 300 secondary metabolites that have weak to very strong antimicrobial activities; however less than half of these compounds appear to possess significant antimicrobial potential[19].

There are many major antimicrobial compounds found in plants (Table 2), like simple phenolics and polyphenols, terpenoids, alkaloids, lectins and other compounds[20].

Class	Subclass	Example(s)	Mechanism	Reference(s)
Phenolics	Simple phenols	Catechol	Substrate deprivation	174
		Epicatechin	Membrane disruption	226
	Phenolic acids	Cinnamic acid		66
	Quinones	Hypericin	Bind to adhesins, complex with cell wall, inactivate enzymes	58, 114
	Flavonoids	Chrysin	Bind to adhesins	175, 182
	Flavones		Complex with cell wall	
		Abyssinone	Inactivate enzymes	32, 219
			Inhibit HIV reverse transcriptase	164
	Flavonols	Totarol	?	122
	Tannins	Ellagitannin	Bind to proteins	196, 210
			Bind to adhesins	192
			Enzyme inhibition	87, 33, 35
			Substrate deprivation	
			Complex with cell wall	
			Membrane disruption	
			Metal ion complexation	
	Coumarins	Warfarin	Interaction with eucaryotic DNA (antiviral activity)	26, 95, 113, 251
Terpenoids, essential oils		Capsaicin	Membrane disruption	42
Alkaloids		Berberine	Intercalate into cell wall and/or DNA	15, 34, 73, 94
		Piperine		
Lectins and polypeptides		Mannose-specific agglutinin	Block viral fusion or adsorption	145, 253
		Fabatin	Form disulfide bridges	
Polyacetylenes		8S-Heptadeca-2(Z),9(Z)-diene-4,6-diyne-1,8-diol	?	62

Table 2: Major classes of antimicrobial compounds from plants[20]

Bay Leaf (*Laurus nobilis*)

Classification

Kingdom: Plantae

Division: Mangoliophyta

Class: Mangoliopsida

Order: Laurales

Family: Lauraceae

Genus: *Laurus*

Species: *nobilis*

Figure 3: A/ *Laurus nobilis* tree. B/ *Laurus nobilis* fresh leaves and flowers. C/ Dried leaves of *Laurus nobilis*.

According to Webster's online dictionary (http://www.websters-online-dictionary.org) bay leaf (Figure 3) is also known as true laurel, sweet bay, Grecian laurel, laurel leaf, or bay tree. It is an evergreen aromatic shrub or tree that could reach 10-18m tall, and is native to the Mediterranean area. It has an olive green or reddish blue color. The evergreen leaves are mixed with short stalks, lanceolate or lanceolate oblong, acuminate. The leaves are about 5-8cm long and 3-4cm wide. The leaves are evergreen when fresh and are used mainly in food, where its dried leaves and essential oils are used in soups and seasoning[21]. Bay leaf has important cultural significance to many countries. For example, it is engraved on the Japanese 10 yen coin, and it had a large significance in ancient Greece, where they used to crown the heads of champion athletes with bay leaves.

As mentioned above, bay leaf also has a very important role in culinary preparations. It has not been reported to have much importance in traditional medicine, but recently it has been subjected to scientific studies examining its molecular constituents. Some scientists believe that food can be a great modification method for disease prevention, also commonly known as complementary or alternative medicine (CAM)[22].

Traditionally bay leaves and fruit were used as treatments for hysteria and emmenagogues (herbs that stimulate blood flow in the pelvic area and uterus; some stimulate menstruation). Infusions were made of bay fruit powder for diuretic and carminative (prevent or remove flatulence) properties. Furuncles, sprains, bruises and rheumatism were also treated externally with fatty oils from the bay fruit and were also

used as insect repellent. Some scientific papers presented evidence that essential oils of bay leaves have anticonvulsant, analgesic and anti-inflammatory activities[23,24,25].

In this study, with the aid of organic solvents, we obtained crude extracts from bay leaves. We wanted to test the cytotoxic effect of bay leaf crude extracts on cell cultures. The cell lines that we used were: human embryonic kidney (CRL-11268-ATCC), human lung fibroblast (CCL-75-ATCC), HeLa (human cervical adenocarcinoma CCL-2-ATCC), and human breast adenocarcinoma (HTB-22-ATCC). Cytotoxicity testing was done using the alamarBlue® assay and release of lactate dehydrogenase (LDH) to screen for cell viability and prolifiration. Also, we examined the possibility of antimicrobial activity against the bacterium *Escherichia coli* (ATCC 25922) using the standard disc diffusion assay. This study was done to determine whether that widely known plants that are usually used as spices and flavorings might be developed into anticancer drug treatments and/or antimicrobial agents.

II. METHODS

Extraction of Plant Material

A modified version of the extraction process of Abdul M *et al* was used[26]. Leaves of *Laurus nobilis* were obtained dried. The leaves were bought from a local supermarket. Ten grams of dried leaves were crushed into small pieces. Five grams of the plant material were then put in each of two flasks. 100 ml of absolute methanol was added to the first flask. Then the mix was ground into fine powder with the homogenizer. Another 150 ml of methanol was added later for the mix; so that the total amount of methanol was used for the 5g of plant material was 250 ml. The same process was done to the second flask except ethanol was used for the extraction.

The mix was left to stir for five days at 230 rpm. Based on previous work, this was believed to be the optimal amount of time needed for the bioactive compounds to dissolve in the organic solvent. Also, methanol and ethanol were used because they have the capacity to dissolve compounds with different polarities. After five days, the mixture was sonicated for two hours using 70 for power and 20 for pulse. The sonication helps improve the extraction of the compounds from the plant.

The resulting mix was filtered with the help of vacuum using two types of filter paper; Whatman filter paper with 0.45 microns pore size, and then Millipore membrane filters with a pore size of 0.22 microns. After filtration, the solvent was evaporated using a rotary evaporator at 64°C. After formation of the crude extract in the form of a film, it

was dissolved in 40 ml of the solvent that it was extracted in. The extract was then distributed into two vials, each containing 20 ml. Both methanol and ethanol extract had two vials. The vials were left for several days until the solvent had completely evaporated, and an air source was used to help speed up the process.

The extraction process was repeated a couple of times using only methanol as solvent. Once the extraction was performed using 10g of plant material and another time using 1g of plant material. Since the amount 1g is so small, adjustments had to be made to accommodate that. It was dissolved in 50 ml of methanol, and the sonication criteria were changed to 20 for the power and 10 for the pulse.

Liquid Chromatography

The International Union of Pure and Applied Chemistry (IUPAC) defines chromatography as follows:

'Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase), while the other (the mobile phase) moves in a definite direction. A mobile phase is described as "a fluid, which percolates through or along the stationary bed in a definite direction". It may be a liquid, a gas or a supercritical fluid, while the stationary phase may be a solid, a gel or a liquid. If a liquid, it may be distributed on a solid, which may or may not contribute to the separation process.'

A chromatographic system consists of a device to place the sample, a mobile phase, a stationary phase, and a detector[27].

1 ml of dissolved extract in methanol was sent for analysis after filtering using a Whatman filter paper with 0.45 micron pores. Liquid chromatographic analysis was used

to determine the molecular constituents. The Analytical Core Lab at KAUST performed this step.

Cyclon P (0.5 x 50 mm) was used as column 1 and Zorbax C18 (150 x 4.6 mm, 3.5 um) as column 2 (Agilent Technologies). The loading solvents that were used were aqueous ammonium bicarbonate (10mM) as solvent A, and 0.1% formic acid in acetonitrile as solvent B. The elution solvents were 0.1% formic acid in acetonitrile as solvent C, and 0.1% formic acid in water as solvent D (raw data not shown).

Antimicrobial Screening

Luria Bertani (LB) Broth Preparation

According to the manufacturing protocol, 35g of LB agar powder (Sigma-Aldrich) was dissolved in 1l of distilled water with no antibiotics added. LB agar contains tryptone, yeast extract, NACL, and agar. Then it was heated while stirring to make sure that all the ingredients dissolved. After that it was autoclaved for 15 minutes at 121°C to sterilize it.

Agar Plates

Forty grams of agarose (Sigma-Aldrich) were dissolved in distilled water, then autoclaved for 15 minutes at 121°C. The medium was then poured into petri dishes and allowed to harden. No antibiotics were added because we wanted to test the antimicrobial activity of the crude extract.

Inoculum Preparation

The method that is used here is a modified version of that used by Kramer J (1961)[28]. The direct colony suspension method was used. This method involves taking a clean tip to take a swab of the bacterial colonies in an agar plate that was then incubated for 18-24 hours. The bacterium that we used to see the effect of the crude extract was *E. coli* (ATCC 25922). The bacterial colonies were subsequently transferred to a tube containing 5 ml LB broth and mixed well. The broth/bacterial mix appeared turbid. After that, 250 µl was taken from the broth/bacterial mix and poured on the agar plates. With the use of a disposable sterile plastic spreader, the bacterial solution was spread on the surface of the agar plate. Spreading the bacterial solution was done next to a fire source to prevent contamination.

Preparation of the Discs

Using Whatman filter papers with 0.45 micron pore size, 8 mm discs were prepared for the antimicrobial assay. At first, 1g of extract was dissolved in 1ml of tap water. Since we are looking into antibacterial activity, the purity of the water was not a serious concern.

Because the crude extract was hard to dissolve, sonication was also used. The crude extract was sonicated for 1 hour in a 60°C heated water bath. For a control, we used our stock of ampicillin at a concentration of 100mg/ml, and kanamycin at a concentration of 50mg/ml. After that, discs were soaked in the dissolved extract and the controls for 30 minutes.

Application of the Discs to the Inoculated Agar Plate

It was important that the length of time between inoculating the agar plate and application of the discs was not too long. Also, we made sure that there was enough space between the discs to allow quantitation of the antibacterial effect. When applying the discs, we also made sure that they were pressed properly onto the surface of the agar plate to make sure that they were in complete contact with the bacteria.

The plates were left for an hour to allow the diffusion of the sample before inverting them and placing them in a 37°C incubator for an additional 24 hours.

The test was repeated multiple times to get statistically reliable results and control for contamination in different extracts. The concentration of the crude extract that was used for soaking the disc was 500mg/ml, dissolved in distilled water with 5% DMSO. This concentration of dimethyle sulfoxide (DMSO) had no observable antibacterial activity. Also, the discs were soaked in the extract overnight to make sure that the discs absorb the bioactive molecules properly, and the amount of bacterial broth was changed from 250 µl to 500µl because when the bacterial broth was in lesser amounts the bacterial lawn that was produced didn't grow properly.

Cytotoxicity Screening

Cell Culture Preparation for AlamarBlue® Assay

The guidelines provided on the company's website were followed (http://www.invitrogen.com). The cell lines were seeded in Dulbecco's Modified Eagle's

Medium (DMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Using a counting chamber, the cell density was measured to be 200,000 cell/ml in a 96 well-plate. The plate was incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. After 48 hours, the cells were treated with the extract. 1g of extract was dissolved in 1ml media then filtered with a 0.8 µm filter. The extract was diluted again with media to get the desired concentration, which was 100 µl. After adding the extract to the cells, the plate was incubated for 24 hours. At the end of this period, 10% of the alamarBlue® reagent was added to the sample volume. The results were obtained by comparing the treated cells with the untreated cells (blanks). Also, to get a better understanding of the cytotoxic activity of *Laurus nobilis* extracts, their results were compared to other plants extracts (data not shown).

Reading the Results

The absorbance of each well containing a particular cell line was measured using a spectrophotometer at 570nm (to monitor absorbance) and 600nm (as a reference wavelength). The readings were done at intervals after adding the dye: 2, 7, 24, and 48 hours.

Statistical Analysis

Cell survival was measured as a percentage of the absorbance, and the data that was obtained with the spectrophotometer was compared against the untreated cells using Microsoft® Excel that measured the standard deviation.

Cell Culture Preparation for Lactate Dehydrogenase (LDH) Assay

We referred to the manual on the company's website as a protocol (http://www.clontech.com). The cell lines were seeded in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The cell density was of 200,000 cell/ml in a 96 well-plate. The plate was incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. After 48 hours, the cells were treated with the extract. 1g of extract was dissolved in 1ml distilled water and sonicated for 10-30 minutes. The extract was then filtered with a 0.8 µm filter. The extract was diluted again with media to get the desired concentration, which was 400 µl. After adding the extract to the cells, the plate was incubated for 24 hours. At the end of this period, the LDH assay was used. As before, the results were obtained by comparing the treated cells with the untreated cells (blanks) also. And to get a better understanding of the cytotoxic activity of *Laurus nobilis* extracts, their results were compared to other plants extracts as well (data not shown).

Statistical Analysis

Cell survival was measured as a percentage of the absorbance, and the data that was obtained with the spectrophotometer was compared against the untreated cells using Microsoft® Excel that measured the standard deviation. The release of the enzyme, which indicated dead cells, showed greater absorbance than the live cells.

III. RESULTS

Crude Extract from *Laurus nobilis*

The process of extracting bioactive molecules from natural sources is usually done either one of two ways: (i) extracting with organic solvents like methanol, and/or (ii) distillation with water. The crude extract that we used was concentrated after removing excess solvent, and because of the nature of the solvent that was used; only certain products absolutes are retained[29].

The outcome of the distillation process is concentrated volatile fractions, which are known as essential oils. This is a different method than that used in this study. Here we relied on the use of organic solvents despite their well-known shortcomings: (i) most organic solvents are known to be toxic and they are regulated by laws in food, (ii) some important volatile compounds can be lost during evaporation of the solvent, and (iii) the products obtained with the use of these toxic chemical solvents are not usually referred to as ”natural”, which affects their consumer acceptance and market value[29].

The first extraction process that used 5 g of dried leaves in 250 ml organic solvent resulted in a yield of 0.94 g for the methanolic extract and 0.77g for the ethanolic extract.

The second time when 10 g of dried leaves were used with methanol resulted in 2.12 g of crude extract.

The third time when 1g of plant material was dissolved in 50 ml of methanol the final amount of extract was 0.13 g.

Liquid Chromatography Analysis

The separation process that in liquid chromatography (LC) happens when the components of the mixture interact to different degrees with the mobile and/or stationary phases, leading to variations of the time in which the mixture components move from the place where the sample was injected to the place where they were detected[30].

LC is one technique used for separation of a very wide range of organic compounds. It has an advantage over gas chromatography (GC) since the compounds that can be separated can range from small-molecule drug metabolites to peptides and proteins. With the addition of mass spectrometry (MS) data, this helps to increase the specificity of the analysis, since MS is more sensitive and specific than other LC detectors. Also, MS helps in analyzing compounds that lack chromophores and can identify unresolved chromatographic peaks[30].

MS works by ionizing the molecules of the analyte. This leads to sorting and identification of these ions based on mass-to-charge (*m/z*) ratio. The ion source and the mass analyzer control the MS function; both have different types that are altered according to the analysis that is being performed. The ion source's function is to generate the ions while the mass analyzer is used to sort out the ions[30].

As mentioned, there are different types of ion sources. The one that was used in this study was electrospray ionization (ESI) as shown in (Figure 4) and (Figure 6). The ESI process depends on generating analyte ions in solution before the analyte reaches the mass spectrometer[30].

It is important to note that other types of detectors could be used simultaneously with MS to provide more confidence in the results. Here, a ultraviolet (UV) detector is also used in addition to the MS as seen in (Figure 5) and (Figure 7).

We can see from the chromatograms in the figures below that there are peaks that represent molecules with possible activity.

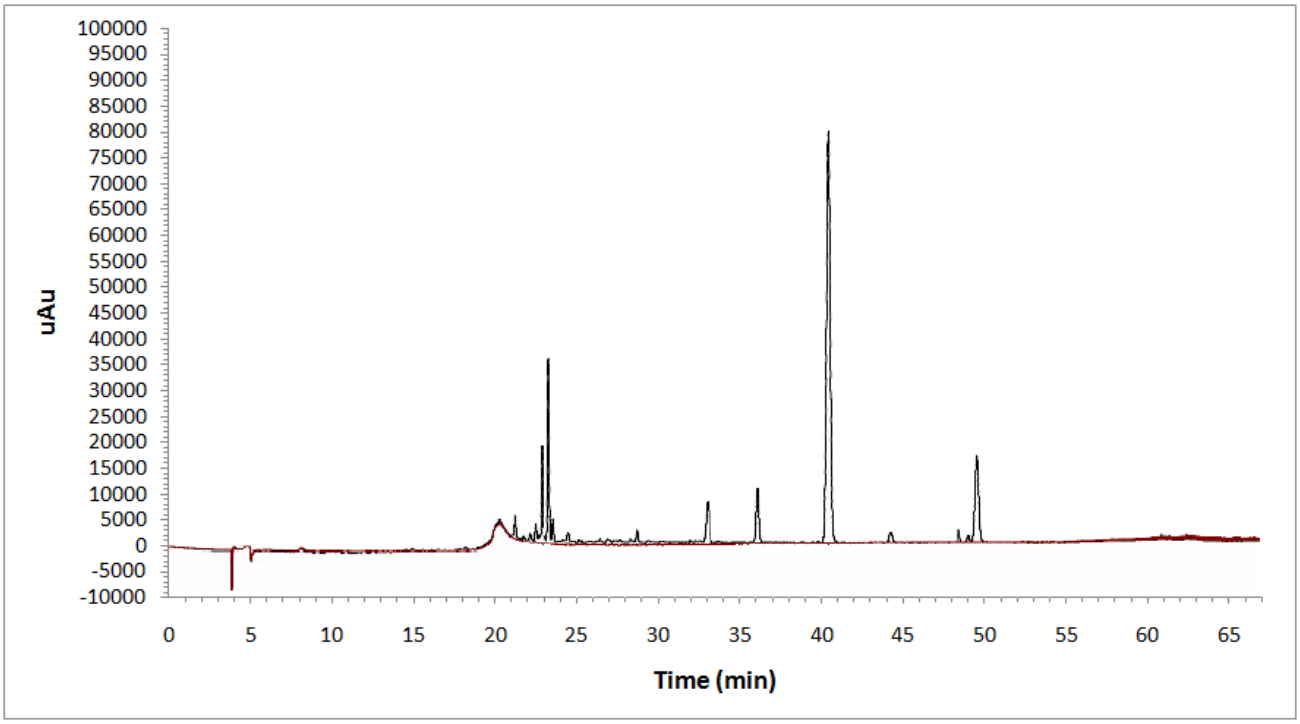

Figure 4: LC-UV chromatogram (λ = 274 nm) of a 2X diluted sample of the methanol extract (black) overlaid over a blank (water/methanol solution; red). The ionization

mode was +ESI. Cyclon P (0.5 x 50 mm) was used as column 1 and Zorbax C18 (150 x 4.6 mm, 3.5 um) as column 2.

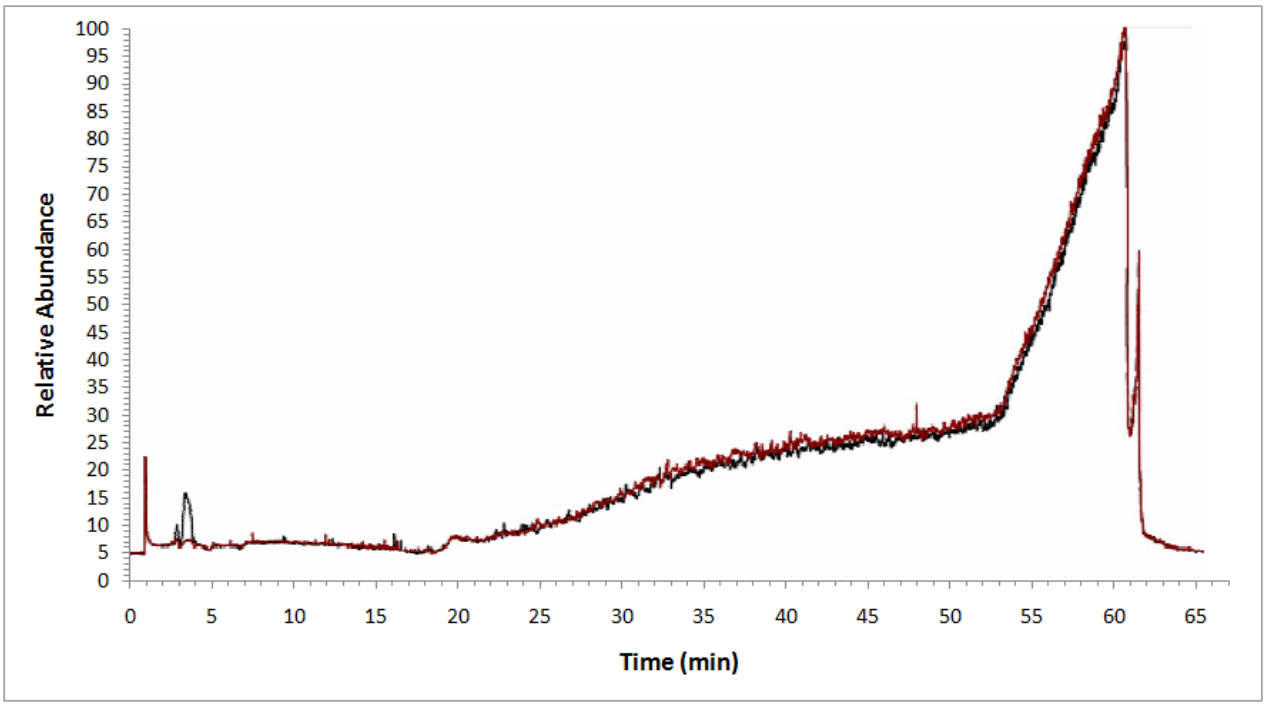

Figure 5: LC-MS total ion chromatogram (acquisition range = 100-1000 amu) of a 2X diluted sample of the methanol extract (black) overlaid over a blank (water/methanol solution; red). The ionization mode was +ESI. Cyclon P (0.5 x 50 mm) was used as column 1 and Zorbax C18 (150 x 4.6 mm, 3.5 um) as column 2.

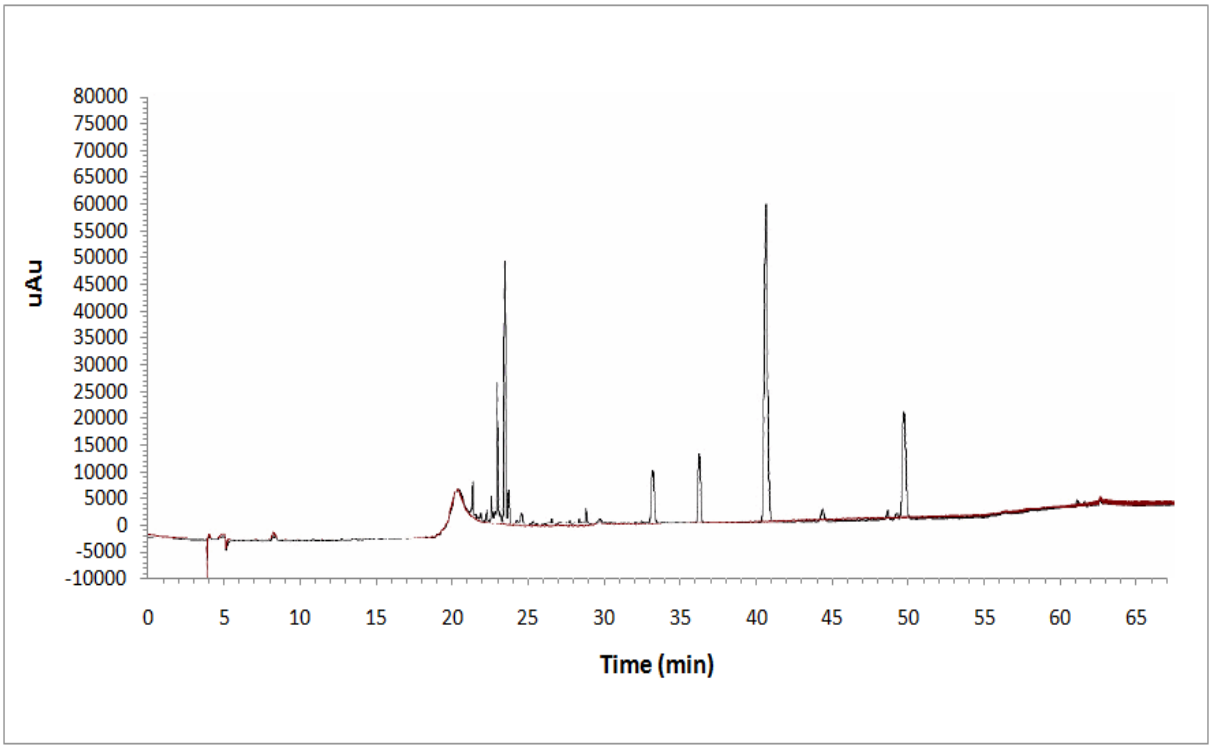

Figure 6: LC-UV chromatogram (λ = 274 nm) of a 2X diluted sample of the methanol extract (black) overlaid over a blank (water/methanol solution; red). The ionization mode was –ESI. Cyclon P (0.5 x 50 mm) was used as column 1 and Zorbax C18 (150 x 4.6 mm, 3.5 um) as column 2.

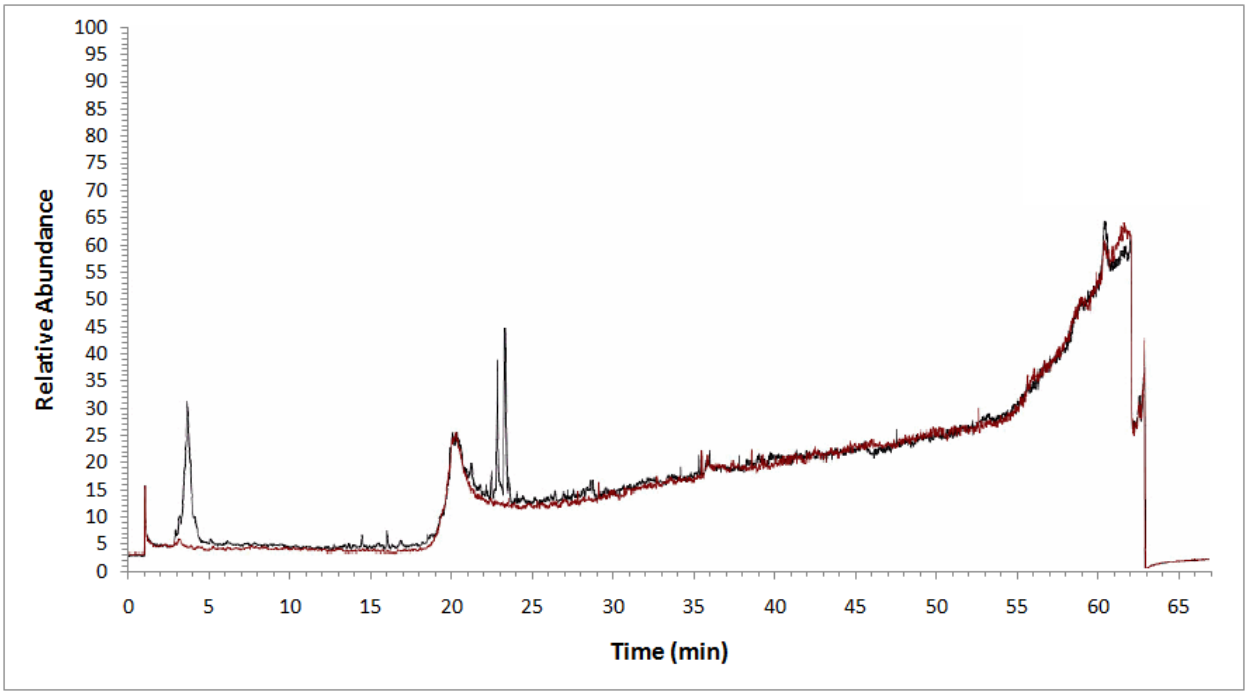

Figure 7: LC-MS total ion chromatogram (acquisition range = 100-1000 amu) of a 2X diluted sample of the methanol extract (black) overlaid over a blank (water/methanol solution; red). The ionization mode was –ESI. Cyclon P (0.5 x 50 mm) was used as column 1 and Zorbax C18 (150 x 4.6 mm, 3.5 um) as column 2.

Antimicrobial Activity

Judging by the naked eye, the inhibition zones that were apparent showed that the crude extract has weak antibacterial activities against *E. coli*. This was evident with the growth of smaller colonies inside the inhibition zone and the small diameter of the inhibition zone compared to the controls that were used, ampicillin and kanamycin. With the aid of a ruler, the inhibition zones were measured (Figure 8).

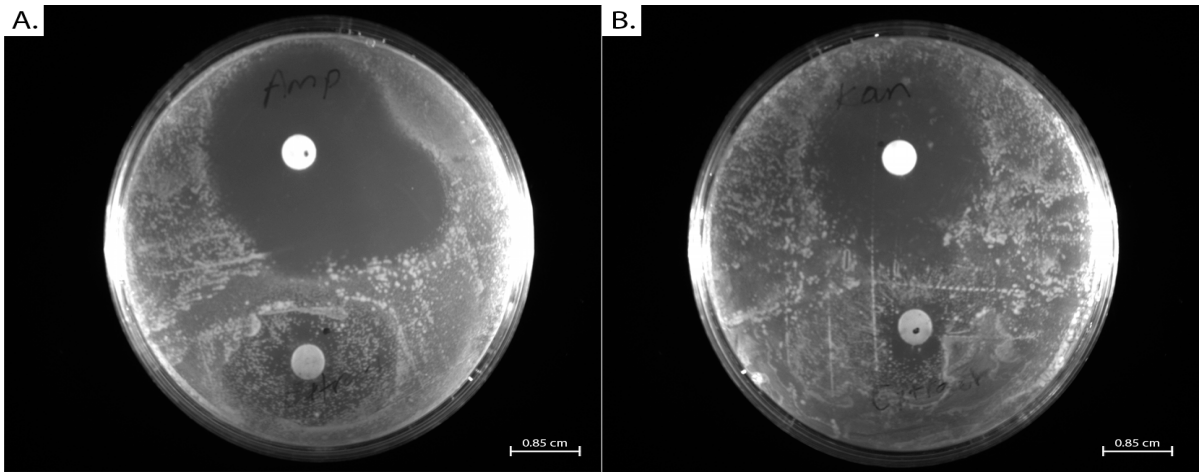

Figure 8: A/ Ampicillin's inhibition zone is measured to be 5.5 cm. The inhibition zone for the 500mg/ml crude extract with 5% DMSO was 1.8 cm, with smaller colonies growing inside the inhibition zone. B/ Kanamycin's inhibition zone is measured to be 4.5 cm. The inhibition zone for the 500mg/ml crude extract with 5% DMSO was 1.4 cm.

Cytotoxic Activity

alamarBlue® assay

According to Invitrogen website (http://www.invitrogen.com), this assay is used to measure cell viability and utilizes the reducing power of the cells to quantitatively measure their proliferation rate. When cells are alive they keep a reducing environment within the cytosol of the cell. Resazurin, the active ingredient in the alamarBlue® reagent, is a non-toxic, cell permeable compound that is blue in color and is non-fluorescent. Upon entering the cell, resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent. alamarBlue® absorbance is measured at 570nm with 600nm as a reference wavelength. By plotting the data acquired in (Table 3), it

appeared that *Laurus nobilis* extract has some cytotoxic activity against breast adenocarcinoma and embryonic kidney cells lines as shown in (Figure 9) when comparing them to untreated cells (blanks).

Cell condition	Absorbance data	Standard deviation
HeLa (Hl) blank	100	0.03
HeLa (Hl) treated	69.7	0.33
Lung fibroblast (LF) blank	100	0.034
Lung fibroblast (LF) treated	105.3	0.24
Breast adenocarcinoma (BA) blank	100	0.26
Breast adenocarcinoma (BA) treated	39.3	0.16
Embryonic kidney (EK) blank	100	0.1
Embryonic kidney (EK) treated	37.3	0.06

Table 3: The quantified data that were obtained from the alamarBlue® assay.

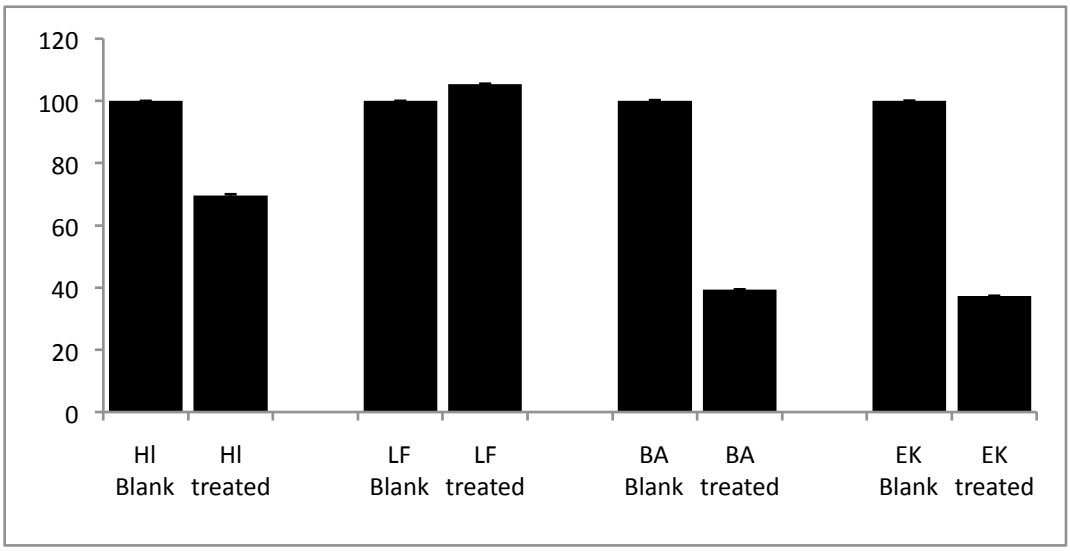

Figure 9: The percentages by which the crude extract was cytotoxic. It shows the comparisons between treated cells and untreated cells (blanks).

LDH Assay

Referring to the manual guide on the company's website (http://www.clontech.com), this assay is a simple colorimetric assay that quantitates cytotoxicity. It is based on LDH activity released from damaged cells into the media caused by damage to the plasma membrane. When LDH is in the media, it reacts with the yellow tetrazolium salt and turns it to a red, formazan-class dye, which is measured by absorbance at 492nm in the case of dead cells. The amount of formazan observed is proportional to the amount of LDH in the culture, which indicates the proportion of damaged or dead cells. In this study, after taking the data from (Table 4) and plotting them, it is shown in (Figure 10) that *Laurus nobilis* extract has effect against embryonic kidney cell line.

Cell condition	Absorbance data	Standard deviation
Blank lung fibroblast (LF)	0.18	0.016
Treated lung fibroblast (LF)	0.37	0.005
Blank embryonic kidney (EK)	0.31	0.05
Treated embryonic kidney (EK)	2.7	0.07
Blank breast adenocarcinoma (BA)	0.13	0.03
Treated breast adenocarcinoma (BA)	0.45	0.02
Blank HeLa (HL)	0.58	0.03
Treated HeLa (HL	1.29	0.12

Table 4: The quantified data that were obtained from the LDH assay.

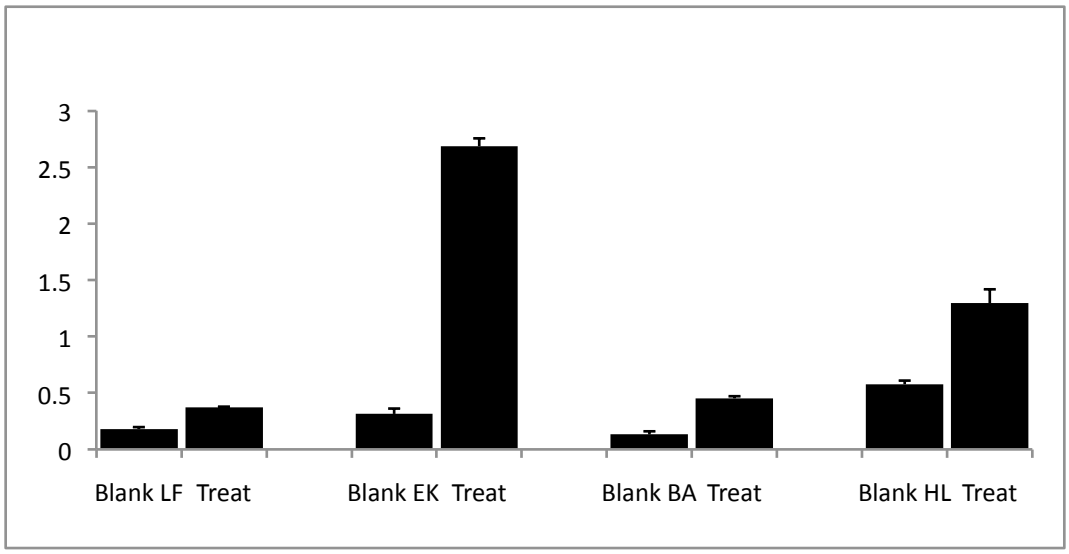

Figure 10: The percentage of release of LDH of which was due to the crude extract's cytotoxic effect against the cell lines. The most significant result was the one against the cell line EK.

IV. DISCUSSION AND CONCLUSIONS

A major goal of medical science is to better the lives of human beings. Phytochemists have been helping in shaping the future by pointing out various applications that could be gained from plants. With the present interest in food chemistry, looking at common plants from a new perspective could significantly impact the drug development industry. Since they are commonly used, they provide us with a potentially useful source of material that is available, safe, and inexpensive[31].

Laurus nobilis has recently been under further scientific investigation. Many of the cytotoxicity reports are based on different parts of *Laurus nobilis.* The assays used have different degrees of sensitivity, based on the cell line that was used for testing. Also, the chemical composition of *Laurus nobilis* has been investigated many times. It has been constant throughout these reports that the chemical composition varies from place to place depending on ecological and environmental elements surrounding the plant.

The volatile oil of bay leaf and other food plants have been prepared and their antiproliferative activity examined. This previous study showed that bay leaf has an antiproliferative activity against human breast adenocarcinoma[31], which was consistent with the finding in this study using the alamarBlue® assay. The chemical composition of essential oils derived from different anatomical parts of *Laurus nobilis* that were collected from different areas in Tunisia was previously examined[32]. Also, the seed oil of *Laurus nobilis* was studied using the methanolic crude extract to search for antimicrobial and antioxidant activities[33]. Another study focused on the radical scavenging activity and

antioxidant capabilities of bay leaf[34]. The process of determining what type of drying method should be utilized when handling bay leaf was also conducted to see the effect on bay leaf's volatile compounds[35].

As mentioned above, numerous studies were conducted to unveil what could possibly be the bioactive molecules contained in bay leaf[36]. Consistent throughout the different reports was that 1,8-cineol (eucalyptol) was one of the major components. The percentage of 1,8-cineol varies according to the place the bay leaf was obtained, but usually it made up ~40% of the volatile oil composition. Other compounds like methyl eugenol and α-terpynil acetate are also found consistently throughout these reports.

A chloroform extract of bay leaf was shown to prominently ameliorate ischemic neuronal death[37]. Another study showed that spirafolide, a compound that was purified from the leaves of *Laurus nobilis,* has neuroprotective effects against dopamine-induced apoptosis[38].

These studies and more suggest that there are potential uses of common plants that could help in guiding us to new discoveries.

The results from this study, and based on previous reports, suggest that bay leaf crude extract exhibits antiproliferative activity. From the results shown here, we could conclude that the mode of action isn't specific to one cell line exclusively. BA and EK cells were both affected in the alamarBlue® assay and EK in the LDH assay.

From a previous report, it was reported that lauroside B, a megastigmane glycoside isolated from *Laurus nobilis* leaves, was able to suppress the proliferation of three human melanoma cell lines, which are highly aggressive tumor cells. The mode of action of lauroside B was shown to be through induction of apoptosis. The authors suggested that the suppression of NF-κB activation is associated with lauroside B induction of apoptosis[39].

It is much too early for us to draw conclusions on the mechanism of action that the crude extract behaved in our studies. But it is an exciting idea that NF-κB might be involved. Testing this hypothesis would be the next logical step in these studies.

NF-κB is a transcription factor known to function in immune regulation and inflammatory responses. Various reports show that deregulation of NF-κB activation was observed in various cancers. Understanding the method by which NF-κB functions in normal and cancer cells led to the observation that a large number of protein kinases stimulate NF-κB activities under different conditions, and some of these kinases are irregularly activated in cancer cells[40].

Regarding the antimicrobial activity that was reported for the extract, it showed a small inhibition zone indicating it has weak activity. Still, we should take into consideration other antimicrobial reports that have been done using *Laurus nobilis* extracts and factor in the ecological and environmental conditions that the plant was exposed to, and how that might affect the chemical composition of the plant extract as was reported before.

As mentioned, the time for making statements regarding the mechanism of how *Laurus nobilis* crude extract works is still far away. With a more in-depth study of *Laurus nobilis*, with the aid of sophisticated fractionation and isolation tools, a better understanding and characterization of the bioactive compounds could be performed. Also, with the help of bioassay derived chemical studies of *Laurus nobilis* extracts could show interesting results that could be beneficial for all of us. On a larger scale, such studies could help shape the view on food chemistry for the future.

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